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A High-Affinity Hemoglobin Is Expressed in the Notochord of Amphioxus, *Branchiostoma californiense*

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In the phylum Chordata, only members of the subphylum Vertebrata were thought to express hemoglobin (Hb). Here we document the existence of intracellular Hb expressed in members of the subphylum Cephalochordata. Hemoglobin is expressed in myotome tissue and in notochord cells within the body of amphioxus. Both notochord and myotome tissue Hbs have a molecular size consistent with a dimeric molecule made up of two non-covalently linked monomers each of approximately 19 kD. The notochord Hb has a relatively high oxygen-binding affinity, with an apparent P_{50} of 0.036 kPa (0.27 mm Hg), and it does not bind oxygen cooperatively. The notochord Hb may be involved in facilitating oxygen delivery and providing a short-term oxygen store within the notochord cells in order to maintain a high level of aerobic metabolism in support of the sustained contraction necessary for notochord function.

Among the metazoa, Hbs are known to exist in members of 12 of the 33 phyla of animals (1,2). In the phylum Chordata, although Hb is nearly universally expressed in members of the subphylum Vertebrata, it has not been identified in any members of the subphyla Urochordata or Cephalochordata. However, these groups of animals share a common ancestor with the vertebrates (3), who inherited the Hb gene from the common ancestor (4,5). Recently, two of us (Doeller and Kraus) observed absorption spectra characteristic of Hb from the notochord of amphioxus from the Gulf of Mexico, *Branchiostoma floridae* (Subphylum Cephalochordata). We verified this observation and also recorded characteristic Hb spectra from both notochord and myotome tissue of another species, *B. californiense*.

The myotome tissue of cephalochordates consists of chevron-shaped bundles of cells, and the notochord tissue consists of a lamellar structure of stacked cells surrounded by a fibrous sheath. The notochord cells are known to have all the features of muscle cells, including the presence of contractile paramyosin fibers, contractility upon stimulation, and neuro-notochordal junctions possessing acetylcholinesterase activity (6,7,8). The stiffening of the notochord was shown to be under neural control, and the paramyosin filaments found in notochord cells contract when myotome filaments contract (6,7). These features clearly demonstrated the contractile nature of the amphioxus notochord, which allows for changes in stiffening that are important for swimming and burrowing in these animals (9).

Cytoplasmic Hb is commonly expressed in contractile cells of vertebrates and invertebrates, where it is often associated with the facilitation of oxygen into the actively respiring cells (10). The expression of Hb in a skeletal support such as the muscular notochord is therefore not unusual. The occurrence of Hb in two species of amphioxus suggests an important function related to the maintenance of the semi-rigid skeleton by supporting muscular activity. This communication presents data identifying

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Abbreviations: Hb, hemoglobin; SEC, size-exclusion chromatography; TFA, trifluoroacetic acid.

the Hb in *B. floridae* and *B. californiense* and describes structural and functional characteristics of the notochord Hb from *B. californiense.*

The *in situ* optical absorption spectra for notochord Hb from *B. californiense* are shown in Figure 1A; Figure 1B shows equivalent spectra for *B. floridae.* Figure 1C shows the *in vitro* optical absorption spectra for myotome tissue crude extracts prepared in 50 m*M* Tris, 100 m*M* NaCl, and 1 m*M* EDTA, pH 7.6. Notochord Hb spectra are shown for each sample equilibrated with air or with 99.999% nitrogen. Myotome Hb spectra are shown for the sample equilibrated with air or under anoxic conditions produced by addition of sodium dithionite. The air-equilibrated notochord samples show absorbance maxima at 418 nm, 546 nm, and 582 nm (±1 nm); the nitrogen-equilibrated samples show absorbance maxima at 435 nm and 556 nm (±1 nm). The dilute *in vitro* myotome Hb spectra show a distinct Soret band at 416 nm (±1 nm) in air and 430 nm (±1 nm) with sodium dithionite; however, the visible peaks were below detectable levels. The observed absorbance maxima and maxima shifts are reversible and are characteristic of the oxygenated and deoxygenated states of a typical Hb (11). Another peak occurs at 500 nm in the *in situ* notochord and *in vitro* myotome spectra, but this peak does not shift upon changes in oxygenation state and disappears following anion-exchange chromatographic purification, indicating that it is not a globin-related absorbance (data not shown). These data clearly demonstrate that Hb capable of reversible oxygen binding is expressed in two species of cephalochordates, thus expanding the known range of expression among chordates to include nonvertebrate chordates.

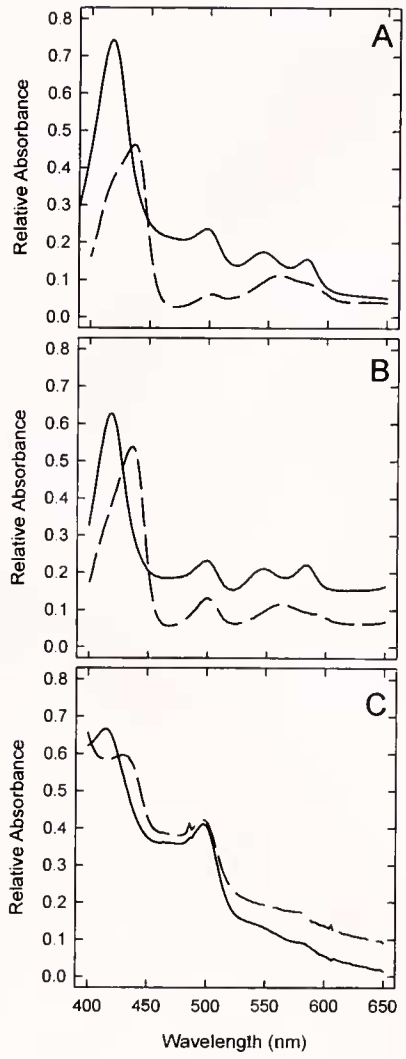

Figure 1. Optical absorption spectra acquired through the anterior region of the isolated notochord of *Branchiostoma californiense* (A) and *B. floridae* (B) equilibrated with air (——) and with 99.999% nitrogen (– – –). *In vitro* spectra of *B. floridae* myotome extracts (C) are shown equilibrated with air (——) and with added sodium dithionite (– – –). Specimens of *B. californiense* were purchased from Pacific Biomarine Supply Co. (Torrence, CA), and those of *B. floridae* were collected from shallow waters on the Gulf of Mexico or purchased from Gulf Specimen Co. (Panacea, FL).

The size-exclusion chromatography (SEC) elution profile at 415 nm for crude protein extract from notochords is shown in Figure 2A and from myotomes in Figure 2B. The crude-extract proteins elute from the SEC column after bovine serum albumin (66 kD) but before cytochrome *c* (12.4 kD). Only the fraction eluting between 8 and 9 min (30–40 kD) in both extracts showed a characteristic Hb absorption spectrum. The anion-exchange elution profile for the Hb-containing SEC fraction from notochords is shown in Figure 2C and from myotomes in Figure 2D. One major peak with a trailing shoulder elutes near the beginning of the gradient, and no other protein peaks are evident. For both notochord and myotome extracts, the first fraction has the characteristic optical absorption spectrum of Hb.

The reversed-phase elution profiles for the Hb-containing anion-exchange fractions are shown for notochord in Figure 2E and myotome in Figure 2F. The chromatograms from the reversed-phase column showed one broad globin band between 10 and 15 min. Even though the chromatograms are off scale in Figure 2E and 2F, the computing integrator indicated two closely eluting bands in the predominant peak in both cases.

Native Hbs from *B. californiense* notochord and myotome tissues elute at about the same time from the size-exclusion column at an equivalent molecular weight of about 38 kD. Additionally, the native Hbs have similar charge densities based on their elution from the anion-exchange column. All of the globin fractions were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis and showed only one band at about 19 kD. This band is of equivalent size for subunits of both noto-

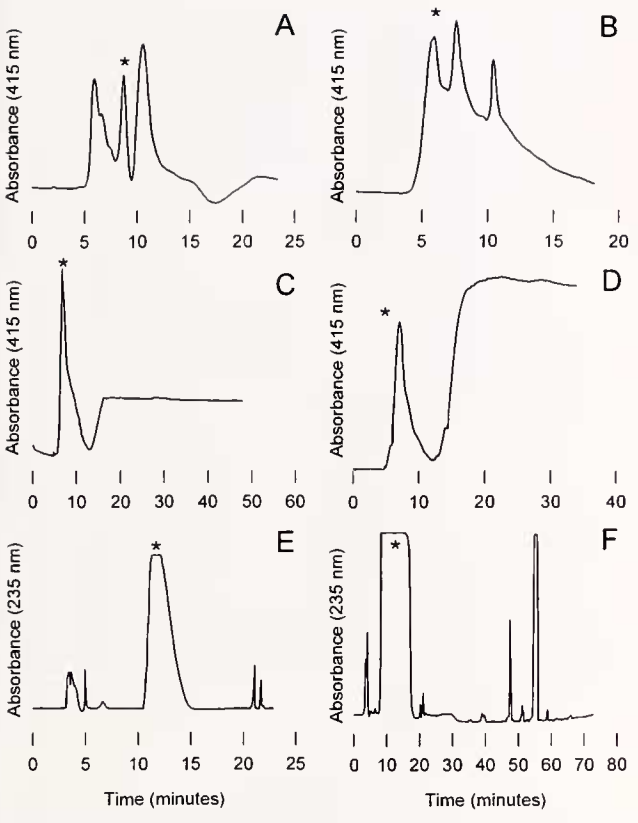

Figure 2. Elution profiles, obtained by high performance liquid chromatography, for extracts of *Branchiostoma californiense* notochord and myotome. Peaks marked with an asterisk represent the Hb-containing fractions. The chromatographic system consisted of a ternary gradient HPLC pump attached to a UV-visible absorption detector via an appropriate column. Size-exclusion chromatography (SEC) was performed on notochord extract (A) or myotome extract (B) using a Polysep-3000 (300 × 7.8 mm) SEC column with a Polysep GFCP (35 × 7.8 mm) guard column equilibrated with 10 m*M* Tris, pH 7.4, at a flow rate of 1.0 ml · min^{-1}. Sample volumes of 0.2 ml were injected and the eluent was monitored at 415 nm. Hb-containing fractions from the size-exclusion column were lyophilized, then resuspended in 10 m*M* Tris, pH 7.4. Anion-exchange chromatography was performed on the Hb fraction from A (C) or the Hb fraction from B (D) on a Biosep DEAE-P (75 × 7.8 mm) column with a Polysep GFCP (35 × 7.8 mm) guard column using a solvent program consisting of a 1-min load with 10 m*M* Tris, pH 7.4, followed by a solvent gradient from 0–80% 10 m*M* Tris/0.5 M sodium acetate in 40 min at a flow rate of 0.5 ml · min^{-1}. Sample volumes of 0.5 ml were injected and monitored at 415 nm. Hb-containing fractions from the anion-exchange column were lyophilized and resuspended in 0.1% trifluoroacetic acid (TFA) for purification by reversed-phase chromatography. Reversed-phase chromatography was performed on Hb fractions from C (E) or Hb fractions from D (F) on a Spherisorb ODS 5 μm d_p (250 × 4.6 mm) column using a solvent program consisting of a 1-min load with 0.1% TFA followed by a solvent gradient from 0–85% 0.1% TFA/acetonitrile in 85 min at a flow rate of 0.8 ml · min^{-1}. Sample volumes of 0.2 ml were injected and the eluent was monitored at 235 nm.

chord and myotome Hb samples. These results, and the reversed-phase chromatographic results, suggest that the notochord and myotome Hbs consist of dimers assembled from two non-covalently linked monomers. It is unknown whether these globins are expressed from the same genes or different genes in the two distinct tissues. The existence of intracellular Hb dimers in cephalochordates is not unexpected because their nearest vertebrate relatives (the lamprey) also produce Hb dimers (12,13,14). Dimers of Hb are common also among the Echinodermata (15,16), a sister taxon to the chordates and a group that probably shares a common ancestor with the chordates (3).

The diameter of the notochord follows the general body taper and, for a *B. californiense* specimen 50 mm in length, is thinner (0.3 mm) toward the ends and thicker (0.6 mm) in the middle. Hemoglobin is detectable in all regions of the notochord, but concentration varies along its length. The heme concentration is approximately 1 m*M* in the thinner anterior and posterior regions of the notochord and 0.5 m*M* in the middle thicker region of the notochord. Concentrations were estimated from absorbance data, determination of the tissue thickness in the region of measurement, and extinction coefficient values for human Hb (11). The oxygen-binding properties of notochord Hb *in situ* were characterized using a computer-controlled Cary Model 14 recording spectrophotometer equipped with a scattered transmission accessory (Aviv Associates, Lakewood, NJ) and a humidified, temperature and gas controlled chamber. Fine forceps were used to completely clean notochord segments of adhering myotome tissue down to the fibrous sheath. The segments were then incubated at 15°C in 0.2 μm filtered 32‰ seawater buffered with 50 m*M* Tris and 10 m*M* KCN at pH 7.4 for 30 min prior to measurements. The technique for determination of O_2-binding equilibria using the scatter transmission system is described elsewhere (17). The Hb from *B. californiense* notochord has a relatively high affinity for oxygen (0.036 kPa, SD 0.013 kPa, n = 5) and no significant cooperativity (Hill coefficient = 1.15, SD 0.14). Oxygen release from the notochord Hb does not occur until the partial pressure of oxygen drops to near 0.25 kPa, and most of the oxygen is released near the P_{50} of the Hb.

The oxygen consumption rates of individual animals were determined with a temperature-controlled, dual closed-chamber polarographic respirometer (Oxygraph 60697, Cyclobios Paar, Graz, Austria) (18). Under normoxic conditions (18.6–20.0 kPa) the oxygen consumption rates for six *B. californiense* individuals ranged from 3.6 to 7.9 μmol $O_2 \cdot g^{-1} \cdot h^{-1}$; under hypoxic conditions (8.6–10.0 kPa), the oxygen consumption rate dropped to between 1.5 and 3.2 μmol $O_2 \cdot g^{-1} \cdot h^{-1}$. Animals were able to initiate swimming motions under hypoxic conditions if disturbed, but otherwise remained quiescent at very low oxygen tensions. All animals survived exposure to the very low oxygen tensions that occurred at the end of the experiments (<0.5 kPa for up to 1 h). In all animals

there appeared to be a general trend of oxyconformity. We tested this phenomenon using the second-degree polynomial (quadratic) model proposed by Mangum and Van Winkle (19). The oxygen tension data (one measurement every 10 s for 6 to 8 h) were standardized as described (19) to millimeters of mercury for X values and fractional oxygen uptake for Y values (ranging from near 0 to 1.0). The data were fit to the quadratic model $Y = B0 + B1X + B2X^2$ by the method of least-squares. The coefficients *B0, B1,* and *B2* were optimized by minimizing the sum of the squared residuals between the polynomial model and the data. The standard deviation about the regression (= SD of the Y estimate) was calculated from the sum of the squared residuals. Two data plots representing the curvilinear extremes and their corresponding quadratic model fits are shown in Figure 3. The standard deviation about the regression for the six *B. californiense* oxygen uptake data sets ranged from 0.018 to 0.085, indicating reasonable fit to the quadratic model. Mangum and Van Winkle (19) found that the *B2* coefficient values for the quadratic model were the most informative concerning responses that deviate from strict oxy-conformity in the direction of oxy-regulation (negative *B2* values) or in the direction opposite to regulation (positive *B2* values). The mean *B2* value for the six animals was near zero (2.40×10^{-5}, range -2.99×10^{-6} to 1.24×10^{-4}); however, it often diverged at the ends of the curves (see Fig. 3A), giving slightly negative values (−0.0004) at low PO_2 and slightly positive values (+0.004) at high PO_2. No value for *B2* is very far from zero, suggesting that oxygen uptake in *B. californiense* conforms closely, although not perfectly, to declining oxygen tension.

In animals whose level of aerobic metabolism is highly dependent on PO_2, a cytoplasmic Hb could support tissue-specific aerobic metabolism in the face of declining tissue PO_2. Myofilament contraction within contractile cells causes the intracellular partial pressure of oxygen to drop rapidly as the result of the high energetic demands of contraction (10). Myoglobin in heart muscle cells has been shown to effectively increase O_2 transport to 30 times the amount that can be delivered by diffusion alone (10). Amphioxus notochord Hb, with its high oxygen-binding affinity *in situ* and hyperbolic oxygen saturation curve, may facilitate diffusion of oxygen into the notochord cells (10). Although a lower Hb concentration in the thicker region of the notochord might reduce the amount of facilitated O_2 diffusion, this decrease might also be ascribed to a greater passive stiffness of the larger diameter thick region compared to the thinner regions. When acting as the skeletal support during fast swimming, amphioxus notochord cells maintain high levels of activity in tonic contracture at peak tension (7,9). This high level of activity requires an adequate supply of ATP energy that could be supplied by aerobic metabolism. By providing a constant supply of oxygen to mitochondria in the notochord cells even under active conditions, the notochord Hb may be crucial for the production of ATP by aerobic pathways, thereby supporting the maintenance of a semi-rigid skeleton.

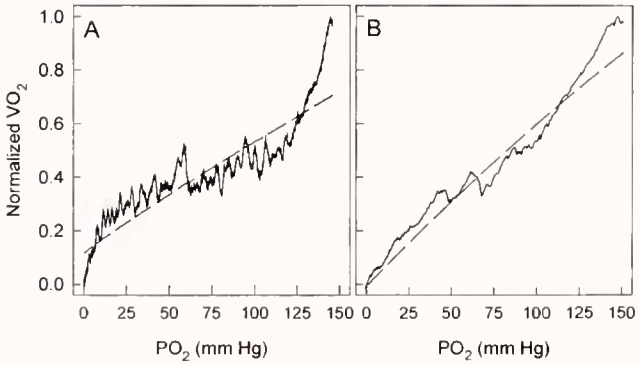

Figure 3. Fractional oxygen uptake of two *B. californiense* single animals (**A** and **B**) plotted as a function of the partial pressure of oxygen. The dashed lines represent the best fit of the data to the second-degree polynomial model proposed by Mangum and Van Winkle (19).

Acknowledgments

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